WO97003197

Publication Title:

No title available

Abstract:

Abstract not available for WO97003197 Data supplied from the esp@cenet database - Worldwide

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PCT

(51) International Patent Classification 6 : C12N 15/31, C07K 14/32, C12N 1/21, 15/67, 15/75 // (C12N 1/21, C12R 1:07)	**A1**	**(11) International Publication Number:** WO 97/03197 **(43) International Publication Date:** 30 January 1997 (30.01.97)

(21) International Application Number: PCT/NL96/00278

(22) International Filing Date: 5 July 1996 (05.07.96)

(30) Priority Data:
95201871.1   7 July 1995 (07.07.95)   EP
(34) Countries for which the regional or international application was filed: NL et al.

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(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

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(54) Title: NOVEL SECRETION FACTORS FOR GRAM-POSITIVE MICROORGANISMS, GENES ENCODING THEM AND METHODS OF USING IT

(57) Abstract

Heterologous protein secretion from Gram-positive bacteria, in particular from Bacilli has, with few exceptions, met with little success. Incompatibility of the heterologous proteins with the protein secretion machinery of the host is the main cause of this effect. This limiting factor for the production of heterologous proteins in commercially significant concentrations from Bacillus subtilis is removed by overexpressing the Bacillus subtilis protein FtsY or FtsY protein in combination with overexpression of other members of the bacterial signal recognition particle. Said gene(s) is(are) overexpressed in Bacillus host cells expressing a heterologous protein which then shows an increased amount of the heterologous protein secreted in the surrounding medium.

Title: Novel secretion factors for Gram-positive
         microorganisms, genes encoding them and methods of
         using it.

This invention relates to processes for the production
of proteins by micro-organisms. Specifically, it relates to
the secretion of heterologous proteins by micro-organisms,
in particular by Gram-positive bacteria, especially by the
5   bacterial host Bacillus.

It also relates to (the overexpression of) a novel gene
encoding a protein involved in the early stages of
prokaryotic protein secretion. Specifically, it relates to
the overexpression of said gene within a Bacillus host
10   (over)expressing heterologous proteins.

B.subtilis and (closely) related bacilli secrete
proteins directly into the growth medium to high
concentrations. Secretion as a mode of production of
proteins of interest, be it homologous to the host or
15   heterologous to the host, be it of recombinant origin or
not, provides several advantages over intracellular
production. It for instance facilitates purification of the
product, it theoretically will lead to a higher yield, no
aggregation of the product will occur, and it gives the
20   possibility for continuous cultivation and production.
However, attempts to secrete heterologous proteins from
B.subtilis and (closely) related organisms at commercially
significant concentrations have, with few exceptions, met
with little success.

25   Nearly all secreted proteins use an amino-terminal
protein extension, known as the signalpeptide, which plays a
crucial role in the targeting to, and translocation of
precursor proteins across the membrane and which is
proteolytically removed by a signalpeptidase during or
30   immediately following membrane transfer. The newly
synthesized precursor proteins are recognized by specific
proteins in the cytoplasm collectively called chaperones.
These chaperones prevent polypeptides, destined for

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translocation, to aggregate or fold prematurely leading to
an export incompatible conformation.

For instance, SecB, GroEL/GroES and DnaK/DnaJ are the
presently known chaperones in the export pathway of <u>E.coli</u>.
5   For the productive binding of precursor proteins to
translocation sites in the cytoplasmic membrane SecA is
needed. SecA, a protein of which cytoplasmic, peripheral as
well as integral membrane forms have been detected, has an
ATPase activity which mediates the initial channelling of
10   precursor proteins into the export pathway.

The SecA subunit acts as a receptor recognizing the
leader and mature domains of the preproteins (Lill et al.
1990) as well as the SecB chaperone (Hartl et al. 1990). It
has been suggested that SecA penetrates into the membrane,
15   after binding of ATP, and so promotes the coinsertion of the
preprotein. After hydrolysis of bound ATP the preprotein is
released from the SecA protein (Schiebel et al. 1991).
Translocation is completed with the proton motive force as
the main driving force and requires members of the integral
20   membrane part of the preprotein translocase complex like
SecY, SecE and SecG (p12/Band1). SecD and SecF are also
integral membrane proteins and are probably participating in
the late steps of protein translocation.

For many years, the protein secretion machinery in
25   prokaryotes has been considered to be independent from the
protein secretion system found in higher eukaryotes (Luirink
et al, 19920. In mammalians, targeting of secretory proteins
to the endoplasmic reticulum (ER) is mediated by the signal
recognition particle (SRP), which is a ribonucleoprotein
30   particle composed of one RNA molecule (SRP 7S RNA) and six
polypeptides of 9, 14, 19, 54, 68 and 72 kD. The SRP
proteins are associated with the RNA as either monomers
(SRP19 and SRP54) or heterodimers (SRP9/14 and SRP68/72).

As soon as the signal peptide of secreted and
35   transmembrane proteins has emerged from the ribosome, it is
recognized and bound by SRP, which also has affinity for the
ribosome. This association slows down the elongation of the

polypeptide chain (elongation arrest). When the complex of
SRP, nascent polypeptide chain, and ribosome bind to the SRP
receptor (SR or docking protein) associated with the ER
membrane, the nascent polypeptide chain is displaced from
5   SRP in a GTP-dependent reaction and protein translation is
resumed.

The translocation of the polypeptide into the ER takes
place co-translationally through a protein pore, the
translocon (Gilmore et al. 1993). Thus, the SRP functions
10 both as a cytosolic chaperone preventing premature folding
of the preprotein by coupling translation to translocation
and as a pilot to guide the preprotein to the SRP receptor
complex in the membrane. The 54kD subunit of SRP (SRP54)
binds to the signal peptide when it emerges from the
15 ribosome and therefore seems to have a key function in the
SRP-mediated process of protein secretion.

To day more and more data become available indicating
that an SRP-mediated export pathway may also function in
other organisms. Homologues of mammalian SRP components have
20 been isolated from Yeast (Hann et al. 1989), E. coli
(Bernstein et al. 1989, and Rymisch et al. 1989), Mycoplasma
mycoides (Samuelsson, 1992) and Bacillus subtilis (Struck et
al. 1989, and Honda et al. 1993).

So it is likely that an SRP-mediated pathway functions
25 in prokaryotes in a separate secretory pathway or may form
part of the general secretory pathway.

In E.coli members of an SRP-like secretory pathway were
identified. These members are Ffh (Fifty four homologue) and
a 4.5S RNA molecule which are homologous to the SRP54 and
30 SRP 7S RNA of eukaryotic SRP (Ribes et al. 1990). It is
shown that Ffh interacts specifically with the signal
sequence of nascent presecretory proteins (Luirink et al.
1992). E.coli protein FtsY, which originally has been
implicated in cell division (because its gene is located in
35 an operon together with FtsE and FtsX) displays striking
sequence similarity with the subunit of mammalian docking
protein. Several observations suggest that FtsY is the

functional E.coli homologue of the mammalian SRP receptor
(Luirink et al. 1994). Depletion of either FtsY, Ffh or the
RNA component of the E.coli SRP affects the export of
several secretory proteins.

5   Also in B. subtilis components of the SRP-like
secretory pathway have been found. The Small Cytoplasmic RNA
(scRNA) was shown to have a functional relationship with the
human SRP 7S RNA and the E.coli 4.5S RNA (Nakamura et al.,
1992). The B.subtilis scRNA is transcribed from the scr gene

10   as a 354 nucleotide precursor which is then processed to a
271 nucleotide RNA at the 5' and 3' end (Struck et al.,
1989), which is similar to its eukaryotic homologue (300
nucleotides) but much larger than the E.coli 4.5S RNA (114
nucleotides). Also the secundary structure of the scRNA is

15   very similar to the eukaryotic SRP 7S RNA, lacking only the
domain III (Struck and Erdmann, 1990). This is in contrast
to the other eubacterial SRP-like RNAs, which only fold into
a single hairpin corresponding to domain IV (Poritz et al.,
1988). Therefore the B.subtilis scRNA is both in size and

20   secundary structure an intermediate between prokaryotic and
eukaryotic SRP-like RNA.

      Besides the scr gene another gene encoding a SRP
constituent has been isolated from B.subtilis. The ffh gene
was found to encode the Ffh protein which shows homology to

25   both the E.coli and eukaryotic SRP54 protein (Honda et al.,
1993).

      It is not unlikely that chaperones or members of the
SRP-like secretion pathway may become a rate-limiting step
in the secretion pathway, the result of which being that the

30   majority of the heterologous protein expressed will
aggregate or fold prematurely. This effect could be the
reason why attempts to secrete heterologous proteins in high
amounts from Gram-positive micro-organisms, in particular
B.subtilis and (closely) related micro-organisms have met

35   with little success. Overexpression of particular members of
the B.subtilis secretion machinery, especially of chaperone-
like proteins which are the rate-limiting step in the

secretion pathway would solve this problem. It is to be
understood that the terms "chaperone" and "secretion factor"
are not completely clearly defined. Both groups of proteins
will at least overlap and in some cases may be identical.

5   Because the mechanism of action of these proteins is not yet
clearly understood, both terms will be used interchangeably
herein.

The invention thus provides a proteinaceous substance
comprising at least a functional part of a chaperone-like

10   protein expressed by Gram-positive bacteria encoded by the
ftsY gene of said bacteria, a representative of said gene
being defined by the sequence of seq. ID no. 7.

When Gram-positive bacteria, especially Bacillus
species and in particular Bacillus subtilis and its closely

15   related organisms are provided with this proteinaceous
substance, of which the functionality is defined as being
able to recognize a protein of interest to be secreted and
lead it into the secretory pathway, they will have an
enhanced capability of secreting proteins. It is very likely

20   that proteins to be secreted must have a signal sequence,
which may be their own signal sequence, or a signal sequence
of a homologous protein of the host bacteria or a signal
sequence homologous to the micro-organism from which the
proteinaceous substance, i.e. the secretion factor according

25   to the invention is derived.

The protein of interest may be any protein which up
until now has been considered for expression in prokaryotes,
as long as it can be provided or has of its own a signal
sequence which render it suitable for secretion in a Gram-

30   positive host. Of course it must also be able to be
recognized (if possibly not very efficiently) and lead into
the secretory pathway by the chaperone-like proteins
according to the invention. It may not be the case that the
chaperone-like proteins would be capable of recognizing and

35   leading into secretion each and every protein by itself.
Other secretion factors may become the rate-limiting step,
if the presently invented secretion factor is provided in

sufficient quantities. In that case it is preferred to also provide the hosts with the secretion factors which may become the rate-limiting step in sufficient quantities also. Since we believe that there is a SRP-like route in Bacillus

5   species and other gram-positive bacteria, it would be advantageous to provide the micro-organism with enhanced amounts of FtsY, the 7S scRNA and Ffh.

The protein of interest may be either homologous or heterologous to the host. In the first case overexpression

10   should be read as expression above normal levels in said host. In the latter case basically any expression is of course overexpression.

The proteinaceous substance according to the invention, which for convenience will often be referred to as the

15   chaperone, secretion factor or the chaperone-like protein, may be homologous to the host, which is preferred, but it · may also be heterologous to the host, as long as it is compatible with the secretion machinery of the host. It stands to reason that this will be most likely in closely

20   related organisms. Thus in the case of a <u>Bacillus subtilis</u> secretion factor, it would be preferred to use it in a <u>Bacillus</u>.

The sequence being depicted as giving a representative of a sequence encoding a chaperone-like protein according to

25   the invention is given in order to enable the person skilled in the art to find homologous sequences which encode similar or functionally the same chaperone-like proteins in other Gram-positive bacteria, in particular of other Bacillus species. Given the general level of skill in the art, it

30   will be routine work to prepare for instance primers based on the given sequence and to screen for other homologous sequences encoding said chaperone-like proteins. These chaperone-like proteins from other related organisms should therefore be considered as part of the present invention.

35   Their DNA and/or amino acid sequences usually will be quite homologous. As a rule the homology will be greater then 70% overall, in particular homologies of greater than 85% ·

overall are to be expected. It is understood that all
homologous genes which can hybridize with the sequence
depicted in seq. ID no. 7 and which encode a protein of
essentially the same structure or function are comprised in
5   this invention. The following equation, which has been
derived from analyzing the influence of different factors on
hybrid stability:

Tm = 81 + 16.6 (log10 Ci) + 0.4 (% G + C) - 600/n - 1.5%
10   mismatch (Ausubel et al., supra) where
            n = length of the shortest chain of the probe
            Ci = ionic strength (M)
            G + C = base composition,
can be used to determine which level of homology can be
15   detected using DNA-DNA hybridisation techniques.
        Therefore the term "essentially of a structure" is
intended to embrace sequences which can include conservative
mutations, where the sequence encodes the same amino acid,
but which may differ by up to 35% in the DNA sequence
20   according to the above equation, more typically by up to
10%. It is not always necessary to have a complete
chaperone-like protein to perform the functions of
recognizing the protein to be secreted and leading said
protein into the secretion pathway. Where possible the
25   hosts may thus also be provided with functional parts of
said secretion factor. It is also possible and even likely
that association with non-protein material such as the 7S
RNA may occur when the secretion factor performs its
functions. The term proteinaceous substance is chosen to
30   include such associations. It is by now well known in the
art that mutations in proteins may lead to higher activity,
longer half-lives, better stability of the mutated protein.
Such derivatives of the secretion factors according to the
invention are also part thereof, since given the information
35   presented herein, it is routine work to find weak spots, or
other sites interesting for mutation in the secretion

factors according to the invention and making site-directed mutations.

A preferred embodiment of the present invention is a proteinaceous substance which is a chaperone-like protein
5   which is at least partly encoded by the ftsY gene of a Bacillus species. Bacillus species are highly preferred organisms to express genes of interest in and a lot of developmental and production experience is available. As stated before however, there has always been a problem with
10   secretion of especially heterologous proteins from Bacillus. This problem may in many cases be solved by providing Bacillus organisms with chaperone-like proteins from other related species, but it will be clear that chances of a good functional secretion factor in Bacillus, including
15   recognition of the heterologous protein are highest using a chaperone-like protein which is derived from a secretion factor in a Bacillus species. Of special interest as a chaperonelike protein is a proteinaceous substance which is at least partly encoded by the ftsY gene of Bacillus
20   subtilis or another Bacillus species. These proteinaceous substances are very likely to be analogues of the eukaryotic docking protein (or SRP receptor) as is the case with products derived from the E.coli ftsY gene. Lack of sufficient amounts of this chaperone-like protein will
25   definitely have a great influence on the capability of host micro-organisms to secrete any proteins, let alone heterologous proteins. At present we believe that heterologous protein may be predominantly secreted using the SRP-like route, i.e. by binding to Ffh, the 7s RNA and the
30   FtsY, whereas homologous proteins use the general secretion pathway. It is also clear that if a heterologous protein to be secreted is provided with a signal homologous or very closely related to a signal present in Bacillus secretory proteins that the presence of a sufficient amount of the
35   secretion factor which normally has the function of recognizing such a signal will lead to enhanced secretion.

The preferred method of providing a Gram-positive
bacteria, in particular a Bacillus species with the
possibility of expressing sufficient amounts of the
chaperone-like proteins (and for instance scRNA) according
5   to the invention is of course by providing said micro-
organism with the genetic information to overexpress said
chaperone-like protein. The invention therefor also provides
a recombinant DNA molecule comprising at least a part of an
ftsY gene encoding a chaperone-like protein of Gram-positive
10   bacteria, said part encoding at least a functional part of
said chaperone-like protein, whereby a representative of
said gene has the sequence of seq. ID no. 7.

As is true for the proteinaceous substances of the
invention the given sequence is given for the reason of
15   enabling the skilled person to find homologous sequences
encoding similar secretion factors. Variants may exist
within Bacillus species and other Gram-positive bacteria. It
will also enable the person skilled in the art to construe
silent mutations, to construe beneficial mutations or
20   mutations having no effect on the activity of the chaperone
resulting from expression. For different species codon
preference may de different, degeneracy may be accounted
for. All these modifications should be considered to be
within the scope of the present invention. To define which
25   genes still belong to the invention can really only be done
by their functionality. If they encode a substance which has
the same activity (in kind, not in amount) as the presently
invented chaperone-like proteins then the gene (or the
recombinant DNA molecule) should be considered to belong to
30   the present invention, if the molecule is derived from a
Gram-positive bacteria , in particular a Bacillus species.
Usually this will coincide with a rather high degree of
homology for instance of 70-95% overall.

A further preferred embodiment of the present invention
35   is of course a gene or a recombinant DNA molecule comprising
at least a part of the _ftsY_ gene of a Bacillus species. The
main reason for this preference is of course that Bacillus

species are well known production organisms in which for
reasons already mentioned it would be helpful to provide an
autologous (sometimes also called homologous) chaperone-like
protein. The most preferred chaperone-like protein at the
5　present time is the one encoded by a recombinant DNA
molecule comprising at least a part of the _ftsY_ gene of
Bacillus subtilis.

　　For easy transfer of the genetic information of the
secretion factors according to the invention it is preferred
10　to provide the recombinant DNA molecule as a vector. The
invention thus also provides a recombinant vector comprising
a recombinant DNA molecule as disclosed above and suitable
regulatory elements for replication and/or expression. The
nature and kind of such a vector is not important, as long
15　as it is capable of transferring the wanted genetic
information into the desired micro-organism and preferably
being capable of replicating or being replicated in such
micro-organism. They may comprise many additional
advantageous features such as marker genes, restriction
20　sites, etc. Chromosomal integration of (part of) the gene
according to the secretion factor is also comprised within
this invention. It would of course be advantageous to only
have to transfer a micro-organism with one vector.
Preferably the invention provides a recombinant vector as
25　described above further comprising a gene encoding a protein
of interest to be secreted.

　　The invention further provides micro-organisms which
have been provided with the genetic information to encode a
chaperone-like according to the invention by whatever
30　method. The invention thus includes a cell derived from a
Gram-positive host cell comprising a recombinant DNA
molecule or a vector as defined herein before. Preferably
the cell is derived from a Bacillus species.

In a further preferred embodiment the cell has also been
35　provided with the ability to overexpress either or both 7S
scRNA and Ffh in a similar manner as it has been provided
with the (over)expression of FtsY.

In a further preferred embodiment the cell also has been provided with the ability to overexpress a homologous protein or to express a heterologous protein. A suitable way to arrive at such a cell is providing it with the genetic

5   information for said protein of interest, leading to a cell comprising a vector having the genetic information encoding a chaperone-like protein according to the invention, further comprising a vector comprising a gene encoding a protein of interest to be secreted. All methods leading to the products

10   of the invention are of course also part of this invention. In particular important are the methods leading to the enhanced production of proteins secreted in the culture medium. The invention thus also includes a method for enhancing the secretion of a protein of interest from a

15   Gram-positive micro-organism, comprising the steps of providing said micro-organism with the possibility to over express the protein of interest, providing the micro-organism with the possibility of overexpressing a proteinaceous substance according to the invention, and

20   culturing said micro-organism under suitable conditions.

Preferably the possibility to overexpress the protein of interest is provided by a vector as disclosed herein before and the possibility to overexpress a proteinaceous substance according to the invention is also provided by a

25   vector as disclosed hereinabove.

The invention will now be further illustrated in the following detailed description and the examples.

DETAILED DESCRIPTION OF THE INVENTION

There is now growing evidence that poor expression and/or secretion is caused by incorrect folding of the heterologous protein in the host cell. The cause of this

5   effect may be the incompatibility of the host cell's chaperone-like proteins of the regular secretory pathway and the heterologous protein. As a result the newly synthesized heterologous proteins will be recognized very inefficiently and in this way become a rate-limiting step in the

10   translocation process. This will be even more pronounced if the heterologous protein is overexpressed. One possibility to overcome this problem is to express heterologous chaperone-like proteins which are homologous to the heterologous protein which is to be secreted. Expression of

15   E.coli SecB in B.subtilis has shown to facilitate secretion of the SecB-dependent maltose-binding protein of E.coli (Collier, 1994). This option is probable not applicable when the heterologous protein and secretion factor are from a more phylogenetic distant organism. In this way the host

20   cell's regular secretion machinery could become incompatible with the heterologous chaperone-like protein itself, leaving the same effect: extreme low secretion efficiency. Another possibility, part of this invention, is to overexpress one or more of the host cell's chaperone-like proteins,

25   preferably the SRP-like chaperone-like proteins and so increase the availability of these chaperone-like proteins for the heterologous protein.

Because homologues of SecA (Sadaie et al. 1991), SecE (Jeong et al. 1993), SecY (Su et al. 1990), and Lep (Van

30   Dijl et al. 1992) have been identified in B.subtilis, it is suggested that signal peptide-dependent protein secretion in B.subtilis utilizes a Sec-pathway that is similar to that of E.coli. So far SecB, which is considered to be the major chaperone in E.coli, seems to be the only chaperone which

35   has a direct binding affinity for SecA and so contributes to the accurate targeting of the preprotein-SecB complex to the membrane bound translocase. The SecB protein is needed for

only a subset of the envelope proteins so SecB independent
proteins will enter the Sec-pathway with the aid of helper
proteins like GroEL/GroES, DnaK/DnaJ or other proteins like
SRP. In eukaryotic organism SRP mainly is responsible for
5   the translocation across the ER membrane. Recently more
evidence has become available of the existence of an SRP
mediated secretion route in bacteria. Because the eukaryotic
pathway has probably evolved from the bacteria it is
thinkable that said proteins are also dependent on this
10   pathway when said proteins are expressed in bacteria like
Bacillus. Thus optimisation of this particularly pathway in
Bacillus will be more profitable for heterologous
(eukaryotic) proteins secretion than the optimisation of the
well known sec-pathway. This invention relates to the
15   cloning of the Bacillus ftsY gene and its effect after
(over)-expression, alone or in combination with other
members of the bacterial SRP, upon heterologous proteins.

For the cloning of B.subtilis ftsY degenerate primers
were synthesized making use of the existing homology boxes
20   between the SRα homologues of different organism (Fig. 2a).
After an inversed PCR reaction using a 110 bp fragment,
which was derived from a nested PCR reaction, as template a
4 kb fragment could be detected. Sequencing results (Fig.
2b) revealed an open reading frame of 329 amino acids. This
25   protein shared 48.2% amino acid identity and 65% similarity
with the ftsY gene of E.coli.

As will be shown in the Examples, the hybridizing
experiments originally lead to an unwanted result, i.e. a
smear of indistinct bands. Surprisingly, we were able by
30   cutting out a region around the expected size of the
amplified fragment and applying PCR to that region again in
resolving this smear into a group of distinct bands.
Unfortunately hardly any band was seen at the expected size
of the fragment that should have been amplified. However, in
35   a third round of amplification we were nevertheless able to
obtain a fragment which could be used further.

EXAMPLE 1

Construction of a Promoter Vector for Secretion factor
Overexpression

pHB201 is capable of autonomous replication in both
5   E.coli (high-copy) and Bacillus (low-copy) strains. This
plasmid confers resistance to the antibiotics
chloramphenicol and erythromycin in E.coli and Bacillus.
Further the plasmid carries a CAT86::lacZ fusion gene
preceded by the strong Lactococcus lactis promoter 59 which
10 also act as strong promoter in B.subtilis (Van der Vossen et
al. 1987).

By replacing the SalI/EcoRI fragment of this plasmid by
a synthetic DNA fragment (SEQ ID NO:1) the CAT86::lacZ was
deleted and an unique NdeI restriction site introduced
15 overlapping the translation initiation site generating
pHBNde (Fig. 1a). This allows us to express the chaperone
genes directly downstream the strong Lactococcal promoter 59
without creating fusion proteins as would be with the
orginal pHB201 vector (Fig. 1b).

15

EXAMPLE 2

Molecular Cloning of _Bacillus subtilis_ DNA Fragments
Homologous With the Human SRα Gene

A set of three Polymerase Chain Reactions (PCR) were
performed as follows. Chromosomal DNA of _B.subtilis_ 168 was
used as template in a first PCR reaction with degenerate
primers AB4229 (SEQ ID NO: 2) and AB4230 (SEQ ID NO: 3).
After 30 cycles and an annealing temperature of 40°C the
amplified DNA was fractionised by electrophoresis on a 2%
Metaphor (FMC BioProducts) agarose gel. The results showed a
smear of ill resoluted bands. DNA fragments ranging in size
from 220 bp to 300 bp were purified from the agarose gel
with a QIAquick gel extraction column (QIAGEN).

1/50 of these isolated fragments were used in a second
PCR with degenerate primers AB4229 and AB 4241 (SEQ ID NO:
4) using the same reaction conditions as in the first PCR.
The result of this PCR showed a number of district bands,
however, a band of the expected size (± 120 bp) was hardly
visible. Fragments of ± 120 bp were isolated from the
agarose gel as above and used in a third PCR with the same
primers and conditions as were used for the second PCR. The
resulting single fragment was isolated, purified and after
treatment with T4 polynucleotide kinase ligated into
dephosphorylated pUC18 linearized with SmaI.

After electroporation to _E.coli_ JM109, selection on
IPTG/X-gal plates, the DNA from six white colonies were used
for automated sequencing.
Within all six isolates an Open Reading Frame (ORF) could be
detected. This ORF showed over 70% similarity with an
alignment of homologs from several other organisms (Fig.
2b).

EXAMPLE 3

Sequencing of Unknown DNA Sequences Adjacent to a Short
Stretch of Known Sequence

By using a labelled internal _ftsY_ fragment derived from
5 example 2 we could detect a single 4 kb PstI band in a
hybridisation experiment. None of the attempts to clone this
fragment directly into pUC were successful, indicating that
cloning of this fragment could be lethal in _E.coli_. An
inversed PCR (IPCR) was used for determination of the
10 sequence.

A total PstI digest of _B.subtilis_ 168 chromosomal DNA
was used as template in the IPCR with primers AB5356 (SEQ ID
NO: 5) and AB5357 (SEQ ID NO: 6). The resulting fragment was
used directly for automated sequencing making use of the
15 same primers.
The sequence of the rest of the PstI chromosomal DNA
fragment located upstream of the primer AB5357 and
downstream of primer AB5357 was determined by automated
sequencing making use of newly developed primers while the
20 sequence was unveiled. The total 4370 bp DNA sequence of the
PstI fragment is shown in Figure 3.

Analysis of the sequence showed the presence of several
Open Reading Frames (ORFs), including the one for FtsY. When
comparing the overall structure of the SRα like proteins and
25 SRP54-like proteins a common domain is evident which
comprises GTP binding boxes (the G-domain, see Figure 4).
Also from this figure it is clear that the _B.subtilis_ FtsY
protein contains only a very short N-terminal domain, in
contrast to the eukaryotic and _E.coli_ homologues. Since the
30 N-terminal domain in those organisms serves as a membrane
anchor it is possible that in _B.subtilis_ FtsY functions with
a different mechanism, and is possibly more chaperone-like
in its action, although it may still be membrane-bound.

Analysis of the sequence showed the presence of several
35 more ORF's, including a truncated ORF showing homology to
the Ffh protein, and a truncated ORF showing homology to

several DNA segregation proteins like the Yeast protein
SMC1.

The chromosomal organisation of the genes in the PstI
fragment is shown in Figure 5.

5

EXAMPLE 4
Effect of FtsY Depletion Upon the Secretion of Heterologous
Proteins

The effects of depletion of FtsY in Bacillus on the
10 processing and/or secretion of heterologous proteins were
studied by placing the chromosomal ftsY gene under control
of the inducible SPAC promoter (Yansura et. al). For this
the N-terminal part of the ftsY gene was cloned into the
multiple cloning site of pDG148 directly downstream the SPAC
15 promoter. The SPAC-ftsY-penP-lacI fragment from the
resulting pDGFtsY' plasmid was recloned into pPPNeo2 making
the final integration construct pNSFtsY' (Fig. 6).

pNSFtsY' is capable of autonomous replication in E.coli
but not in Bacillus. It confers resistance to the antibiotic
20 ampicillin which can be used for selection in E coli and
neomycin for selection in Bacillus. Integration of pNSFtsY'
into the ftsY locus results in a truncated copy of ftsY
(ftsY') under control of the authentic promoter and an
intact copy of ftsY under control of the SPAC promoter.

25   Neomycin resistant pNSFtsY' integrants could be
selected after a protoplast transformation of B.subtilis
168.
Integrants growing in a medium with 0.5 mM IPTG showed
growth characteristics comparable to B.subtilis host lacking
30 the integrated plasmid. The growth rate of integrants did
not decrease after incubation in the absence of IPTG, nor
did the cell morphology.

Effects of depletion of FtsY on protein translocation
were examined by fermentation experiments using hosts
35 expressing heterologous proteins. A pUB110 like vector
containing regulatory sequences of the B.licheniformis or
B.amyloliquefaciens α-amylase gene was used for the

expression of heterologous proteins. Some of the
heterologous proteins were produced to slightly higher
levels in cells cultured in the presence of IPTG, however in
the absence of IPTG the production of the heterologous
5   proteins was not completely abolished.

These effects were unexpected since in E.coli depletion
of FtsY has a profound effect on the cell morphology and
growth rate. Therefore it is possible that there is FtsY
formation despite the absence of IPTG, indicating
10   transcriptional read through. It should be possible to
correct this by insertion of a strong terminator signal
upstream of the SPAC promoter in the integration construct.
To confirm that the absence of FtsY is hazardous to the cell
attempts were made to disrupt the ftsY gene.
15

EXAMPLE 5
Construction of Bacillus strains without a functional ftsY
gene.

Since the experiments described in Example 4 were
20   unexpected in the sense that strain containing the ftsY gene
under the control of the SPAC promoter were still viable,
and showed no clear phenotype in the absence of the inducer
IPTG, we hypothesized that the construct we used was leaky
so that even in the absence of IPTG a small amount of FtsY
25   would be produced. To eliminate the production of FtsY we
tried to disrupt the ftsY gene by insertion of a neomycin
resistance marker. We constructed a plasmid pBHdSFtsYNeo
which harbours the 5' and the 3' end of the ftsY gene
separated by the Neomycin resistance gene in a vector unable
30   to replicate in B.subtilis.

Plasmid pBHdSFtsYNeo was linearized by cutting with
HpaI and used to transform B.subtilis 168 to neomycin
resistance. This should lead to strains having the original
ftsY gene replaced by the fstY::Neo construct via a double
35   cross-over event (see Figure 7). However, we were unable to
select for Neomycin resistant colonies using this linear

DNA, suggesting the possibility that disruption of the _ftsY_ gene is lethal to the cell.

We therefore repeated the transformation experiments with intact, uncut, plasmid DNA. In this case integration of
5 the plasmid into the _ftsY_ gene can take place via a single cross-over event (Campbell type integration), leading to neomycin resistant colonies which have both an intact and a disrupted copy of the _ftsY_ gene present (see Figure 8). We isolated integrant strains, one of which was shown to have
10 the chromosomal organization represented in Figure 8. Since in this strain two copies of the 3' end of the _fstY_ gene are present recombination between them is possible, leading to excision of the plasmid sequences in between and formation of a strain containing only one copy of the _ftsY_ gene,
15 disrupted by the neomycin resistance marker. Despite several attempts we were unable to isolate such recombinant strains, suggesting again that such strains are unviable.

EXAMPLE 6
20 Effect of FtsY Overexpression on the Location of Precursors and Mature Heterologous Proteins

The effects of overexpression of FtsY in Bacillus on the processing and/or secretion of heterologous proteins were studied by placing the complete _ftsY_ gene under control
25 of the constitutive P59 promoter in pHBNde (see Example 1) resulting in the plasmid pHBFtsY. Effects of overexpression of FtsY on protein translocation were examined by pulse-chase experiments using hosts expressing heterologous proteins.

30   _B.Licheniformis_ T399 was transformed with plasmid pLAT-IL3 containing the human Interleukin-3 (h-IL-3) gene expressed from the _B.licheniformis_ α-amylase promoter and provided with the _B.licheniformis_ α-amylase signal sequence, or with the plasmid pLP10-AB containing the prochymosin gene
35 under the same expression signals. The resulting strains T399IL and T399Chy were transformed with plasmid pHBNFtsY

containing the _ftsY_ gene. As a control also both strains
T399IL and T399Chy were transformed with the vector pHBNde.

Single colonies of all strains were inocculated in 5 ml
of medium starvation medium S7+ (including Methionine and
5   Cysteine) and grown at 37°C overnight. Aliquots of 200μl
were inocculated in 5 ml S7- (without Methionine and
Cysteine) medium and grown for another 5 hours.

After growth to OD=0.3-0.7 a sample of 3.2 ml was
centrifuged, washed with S7- medium and resuspended into
10   3.2 ml fresh S7- medium. The sample was incubated for 20
minutes at 37°C and pulsed with 25μ(Ci L- [^{35}S]-Methionine
(>1000 Ci/mmol) per ml during 60 seconds at 37°C. Then a
chase was performed by addition of 50μl (2 mg/ml) L-
Methionine per ml. The chase was stopped at different time
15   points (0, 15, 30, and 60 seconds) by mixing of 600μl of the
reaction with 600μl ice cold 20% TCA, and incubation on ice
for at least 30 minutes. The samples were centrifugated, and
the supernatant was used directly for immuno precipitation,
SDS-polyacrylamide gel electrophoresis and autoradiography
20   using standard protocols.

The cell pellet was washed with 1 ml aceton, and dried.
The cells were resuspended in 50/1 lysis buffer 10 mM Tris
pH8, 25 mM MgCl, 200 mM NaCl, 5 mg/ml lysozyme) and
incubated for 3 minutes at 37°C. After addition of 50μl TES
25   (20 mM Tris, 2 mM EDTA, 2% SDS, pH8) the samples were boiled
for 5 minutes. To the samples was added 900μl of STDT (10
mM Tris, 0.9% NaCl, 1% Triton, 0.5% Sodium deoxycholate,
pH8.2), the mixture was incubated for 15-60 minutes on ice,
and the debris was precipitated. The supernatant was used
30   for immuno precipitation with antiserum raised against h-IL-
3 or Chymosin and subsequent SDS-polyacrylamide gel
electrophoresis and autoradiography using standard
protocols.

Overexpression of FtsY increased the secretion of the
35   mature form of interleukin-3 and the processing of the
precursor of prochymosin.

21

EXAMPLE 7

Effect of Overexpression of Ffh on the Secretion of Human
Interleukin-3.

The _ffh_ gene encoding the _B.subtilis_ homologue of the
5　eukaryotic SRP54 protein was cloned as a PCR fragment
obtained using primers based on the published DNA sequence
(Honda et al, 1993). The gene was cloned into the vector
pHBNde (see Example 1) under the control of the strong
Lactococcal P59 promoter to form plasmid pHBNFfh.

10　　Pulse-chase experiments were performed as described in
Example 6 with _B.licheniformis_ strains containing both
plasmid pHBNffh and plasmid pLAT-IL3 harbouring the human
Interleukin-3 (h-IL-3) gene under the expression and
secretion signals of the _B.licheniformis_ α-amylase gene.

15　　As is shown in Figure 9, the processing of h-IL-3 is
very fast, as no precursor can be detected. The amount of
mature h-IL-3 in the supernatant fraction is in all cases
higher in the samples obtained from the strain overproducing
Ffh compared to the strain containing only the vector
20　plasmid.

EXAMPLE 8

Effects of Overexpression of scRNA on the secretion of
Human Interleukin-3.

25　　The _scr_ gene encoding the scRNA from _B.subtilis_ was
cloned as a PCR fragment using primers based on the
published DNA sequence (Struck et al, 1989) and following
the approach (including the same 5' primer) described by
Nakamura (Nakamura et al, 1992) introducing a HindIII site
30　just upstream of the scr DNA and a SphI site downstream of
the terminator sequence.

The P59 promoter was deleted from the vector pHB210 by
replacement of the AlwNI-SmaI fragment containing the
origin of replication (_ori_) together with the P59 promoter
35　by the AlwNI-PvuII fragment from vector pBR322 containing
only the same _ori_. This new vector pBHk was used to
exchange the EcoRI-PvuII fragment for the EcoRI-BamHI

(blunted by T4 polymerase) containing the SPAC-penP-lacI
cassette from pDG148 to contruct the vector pBHSpac.

The PCR fragment containing the scr gene was digested
with HindIII and SphI and ligated into pBHSpac digested with
5 the same restriction enzymes to contruct plasmid pBHSscr.
This way the scr gene was placed under the control of the
SPAC promoter.

Pulse-chase experiments were performed as described in
Example 6 with B.licheniformis strains containing both
10 plasmid pBHSscr and plasmid pLAT-IL3 harbouring the human
Interleukin-3 (h-IL-3) gene under the expression and
secretion signals of the B.licheniformis α-amylase gene.

Also in this case the processing of h-IL-3 was very
fast, as no precursor could be detected. However, the amount
15 of mature h-IL-3 in the supernatant fraction was in all
cases higher in the samples obtained from the strain
overproducing scRNA compared to the strain containing only
the vector plasmid, or with plasmid pBHSscr in the absence
of the inducer IPTG.
20

EXAMPLE 9
Effects of Simultaneously Overexpressed Signal Recognition
Particle components on the Secretion of Heterologous
Proteins.
25   The effects described in Examples 6, 7 and 8 were even
more pronounced when more than one of the components of the
bacterial signal recognition particle were simultaneously
expressed. For this purposs the ftsY gene was expressed from
the IPTG inducible promoter located in the chromosome as
30 described in Example 4 by addition of 3 mM IPTG, while the
components Ffh or scRNA were expressed from their pHB210
derived vectors described in Examples 7 and 8 above.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

   (i) APPLICANT:
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     (B) STREET: Wateringseweg 1
     (C) CITY: Delft
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     (F) POSTAL CODE (ZIP): 2600 MA

   (ii) TITLE OF INVENTION: Secretion of heterologous proteins in
     Bacillus

   (iii) NUMBER OF SEQUENCES: 8

   (iv) COMPUTER READABLE FORM:
     (A) MEDIUM TYPE: Floppy disk
     (B) COMPUTER: IBM PC compatible
     (C) OPERATING SYSTEM: PC-DOS/MS-DOS
     (D) SOFTWARE: PatentIn Release #1.0. Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

   (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 170 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (genomic)

   (iii) HYPOTHETICAL: NO

   (iii) ANTI-SENSE: NO

   (vi) ORIGINAL SOURCE:
     (C) INDIVIDUAL ISOLATE: AB4296-4303

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GCTCTAGAGT AGATCTGCAG GCTTTAACGT AGGCAAAGCT CAGGGTAGAC TTTGAATGGA   60

CAGAAACATG ACATATCTCT TGAAAGGATG ATTGTGGTGG TGAAAACAGA TAAAATCTCC   120

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(2) INFORMATION FOR SEQ ID NO: 2:

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   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
   (C) INDIVIDUAL ISOLATE: AB4229

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GGMGTSAAYG GMGTSGGMAA   20

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      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
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   (iii) HYPOTHETICAL: NO

   (iii) ANTI-SENSE: NO

   (vi) ORIGINAL SOURCE:
      (C) INDIVIDUAL ISOLATE: AB4230

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GCRSCYGSRC GRWAKRTRTC   20

(2) INFORMATION FOR SEQ ID NO: 4:

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      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: AB4241

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

ARNCKKCCNG CYGTRTC   17

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: AB5356

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TGCCTGTCAT TAAGCAGACG GCAGGAAGCG ATCC   34

(2) INFORMATION FOR SEQ ID NO: 6:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: AB5357

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

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(2) INFORMATION FOR SEQ ID NO: 7:

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        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iii) HYPOTHETICAL: NO

    (iii) ANTI-SENSE: NO

    (v) FRAGMENT TYPE: N-terminal

    (vi) ORIGINAL SOURCE:
        (A) ORGANISM: B. subtilis
        (B) STRAIN: 168

    (ix) FEATURE:
        (A) NAME/KEY: CDS
        (B) LOCATION: 19..1008
        (D) OTHER INFORMATION: /function= "protein secretion
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           /product= "FtsY"
           /gene= "ftsY"

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GAGGAAAGAG GTTAAAAG ATG AGC TTT TTT AAA AAA TTA AAA GAG AAA ATC   51
               Met Ser Phe Phe Lys Lys Leu Lys Glu Lys Ile
                1   5   10

ACA AAA CAG ACA GAT TCC GTA TCT GAA AAG TTT AAG GAT GGC CTT GAA   99
Thr Lys Gln Thr Asp Ser Val Ser Glu Lys Phe Lys Asp Gly Leu Glu
     15   20   25

AAA ACA AGA AAC TCC TTT CAA AAC AAA GTG AAT GAT CTT GTA TCC CGT   147
Lys Thr Arg Asn Ser Phe Gln Asn Lys Val Asn Asp Leu Val Ser Arg
   30   35   40

TAC CGT AAA GTG GAT GAG GAT TTC TTC GAA GAG CTT GAA GAG GTT CTT   195
Tyr Arg Lys Val Asp Glu Asp Phe Phe Glu Glu Leu Glu Glu Val Leu
   45   50   55

ATC AGC GCG GAT GTC GGT TTT ACA ACC GTT ATG GAA TTA ATA GAT GAG   243
Ile Ser Ala Asp Val Gly Phe Thr Thr Val Met Glu Leu Ile Asp Glu
60   65   .   70   75

CTG AAA AAA GAA GTC AAA CGC AGA AAT ATT CAA GAT CCA AAG GAA GTC   291
Leu Lys Lys Glu Val Lys Arg Arg Asn Ile Gln Asp Pro Lys Glu Val
       80   85   90

AAG TCA GTG ATT TCT GAG AAA CTG GTC GAG ATT TAT AAC AGC GGA GAT   339
Lys Ser Val Ile Ser Glu Lys Leu Val Glu Ile Tyr Asn Ser Gly Asp
       95   100   105

GAG CAA ATT TCA GAA CTG AAC ATC CAG GAT GGG CGT TTA AAC GTA ATC   387
Glu Gln Ile Ser Glu Leu Asn Ile Gln Asp Gly Arg Leu Asn Val Ile
      110   115   120

CTT CTG GTA GGT GTA AAC GGC GTC GGG AAA ACA ACA ACG ATC GGA AAG   435
Leu Leu Val Gly Val Asn Gly Val Gly Lys Thr Thr Thr Ile Gly Lys
     125   130   135

CTT GCT CAT AAA ATG AAA CAA GAA GGA AAA TCT GTT GTA CTT GCC GCC   483
Leu Ala His Lys Met Lys Gln Glu Gly Lys Ser Val Val Leu Ala Ala
140   145   150   155

GGA GAC ACT TTT AGA GCG GGA GCC ATT GAA CAG CTG GAA GTA TGG GGA   531
Gly Asp Thr Phe Arg Ala Gly Ala Ile Glu Gln Leu Glu Val Trp Gly
        160   165   170

GAG CGT ACA GGA GTG CCT GTC ATT AAG CAG ACG GCA GGA AGC GAT CCG   579
Glu Arg Thr Gly Val Pro Val Ile Lys Gln Thr Ala Gly Ser Asp Pro
       175   180   185

GCG GCT GTC ATC TAC GAT GCT GTT CAT GCT GCG AAA GCA AGA AAT GCC   627
Ala Ala Val Ile Tyr Asp Ala Val His Ala Ala Lys Ala Arg Asn Ala
      190   195   200

GAT GTA TTA ATT TGT GAT ACG GCA GGG CGT CTC CAA AAC AAA GTA AAT   675
Asp Val Leu Ile Cys Asp Thr Ala Gly Arg Leu Gln Asn Lys Val Asn
     205   210   215

CTC ATG AAA GAG CTT GAA AAA GTA AAA CGT GTT ATC GAA AGA GAA GTT   723
Leu Met Lys Glu Leu Glu Lys Val Lys Arg Val Ile Glu Arg Glu Val
220   225   230   235

CCT GAA GCT CCG CAT GAG GTG CTG CTT GCC CTT GAT GCC ACG ACC GGC   771
Pro Glu Ala Pro His Glu Val Leu Leu Ala Leu Asp Ala Thr Thr Gly
        240   245   250

CAA AAT GCA ATG GCT CAG GCA AAA GAA TTC TCT AAA GCA ACA AAT GTT   819
Gln Asn Ala Met Ala Gln Ala Lys Glu Phe Ser Lys Ala Thr Asn Val
       255   260   265

30

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ACC GGC ATT GCT TTA ACG AAG CTT GAC GGT ACG GCA AAA GGC GGT ATC    867
Thr Gly Ile Ala Leu Thr Lys Leu Asp Gly Thr Ala Lys Gly Gly Ile
    270         275         280


GTC CTT GCG ATT CGC AAC GAG CTT CAC ATC CCG GTT AAA CTA GTC GGT    915
Val Leu Ala Ile Arg Asn Glu Leu His Ile Pro Val Lys Leu Val Gly
    285         290         295


TTA GGA GAA AAA GTT GAT GAC CTT CAG GAA TTT GAT CCA GAA TCC TAT    963
Leu Gly Glu Lys Val Asp Asp Leu Gln Glu Phe Asp Pro Glu Ser Tyr
300         305         310         315


GTG TAC GGA CTC TTT TCA GAT TTA GTG GAA AAA GCC GAC GAT TAAGAAAAAG   1015
Val Tyr Gly Leu Phe Ser Asp Leu Val Glu Lys Ala Asp Asp
        320         325         330


G                                      1016
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(2) INFORMATION FOR SEQ ID NO: 8:

    (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 329 amino acids
    (B) TYPE: amino acid
    (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: protein

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

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Met Ser Phe Phe Lys Lys Leu Lys Glu Lys Ile Thr Lys Gln Thr Asp
 1        5          10          15


Ser Val Ser Glu Lys Phe Lys Asp Gly Leu Glu Lys Thr Arg Asn Ser
        20          25          30


Phe Gln Asn Lys Val Asn Asp Leu Val Ser Arg Tyr Arg Lys Val Asp
    35          40          45


Glu Asp Phe Phe Glu Glu Leu Glu Glu Val Leu Ile Ser Ala Asp Val
    50          55          60


Gly Phe Thr Thr Val Met Glu Leu Ile Asp Glu Leu Lys Lys Glu Val
65          70          75          80


Lys Arg Arg Asn Ile Gln Asp Pro Lys Glu Val Lys Ser Val Ile Ser
        85          90          95


Glu Lys Leu Val Glu Ile Tyr Asn Ser Gly Asp Glu Gln Ile Ser Glu
        100         105         110
```

Leu Asn Ile Gln Asp Gly Arg Leu Asn Val Ile Leu Leu Val Gly Val
  115   120   125

Asn Gly Val Gly Lys Thr Thr Thr Ile Gly Lys Leu Ala His Lys Met
 130   135   140

Lys Gln Glu Gly Lys Ser Val Val Leu Ala Ala Gly Asp Thr Phe Arg
145   150   155   160

Ala Gly Ala Ile Glu Gln Leu Glu Val Trp Gly Glu Arg Thr Gly Val
  165   170   175

Pro Val Ile Lys Gln Thr Ala Gly Ser Asp Pro Ala Ala Val Ile Tyr
  180   185   190

Asp Ala Val His Ala Ala Lys Ala Arg Asn Ala Asp Val Leu Ile Cys
 195   200   205

Asp Thr Ala Gly Arg Leu Gln Asn Lys Val Asn Leu Met Lys Glu Leu
 210   215   220

Glu Lys Val Lys Arg Val Ile Glu Arg Glu Val Pro Glu Ala Pro His
225   230   235   240

Glu Val Leu Leu Ala Leu Asp Ala Thr Thr Gly Gln Asn Ala Met Ala
  245   250   255

Gln Ala Lys Glu Phe Ser Lys Ala Thr Asn Val Thr Gly Ile Ala Leu
  260   265   270

Thr Lys Leu Asp Gly Thr Ala Lys Gly Gly Ile Val Leu Ala Ile Arg
  275   280   285

Asn Glu Leu His Ile Pro Val Lys Leu Val Gly Leu Gly Glu Lys Val
 290   295   300

Asp Asp Leu Gln Glu Phe Asp Pro Glu Ser Tyr Val Tyr Gly Leu Phe
305   310   315   320
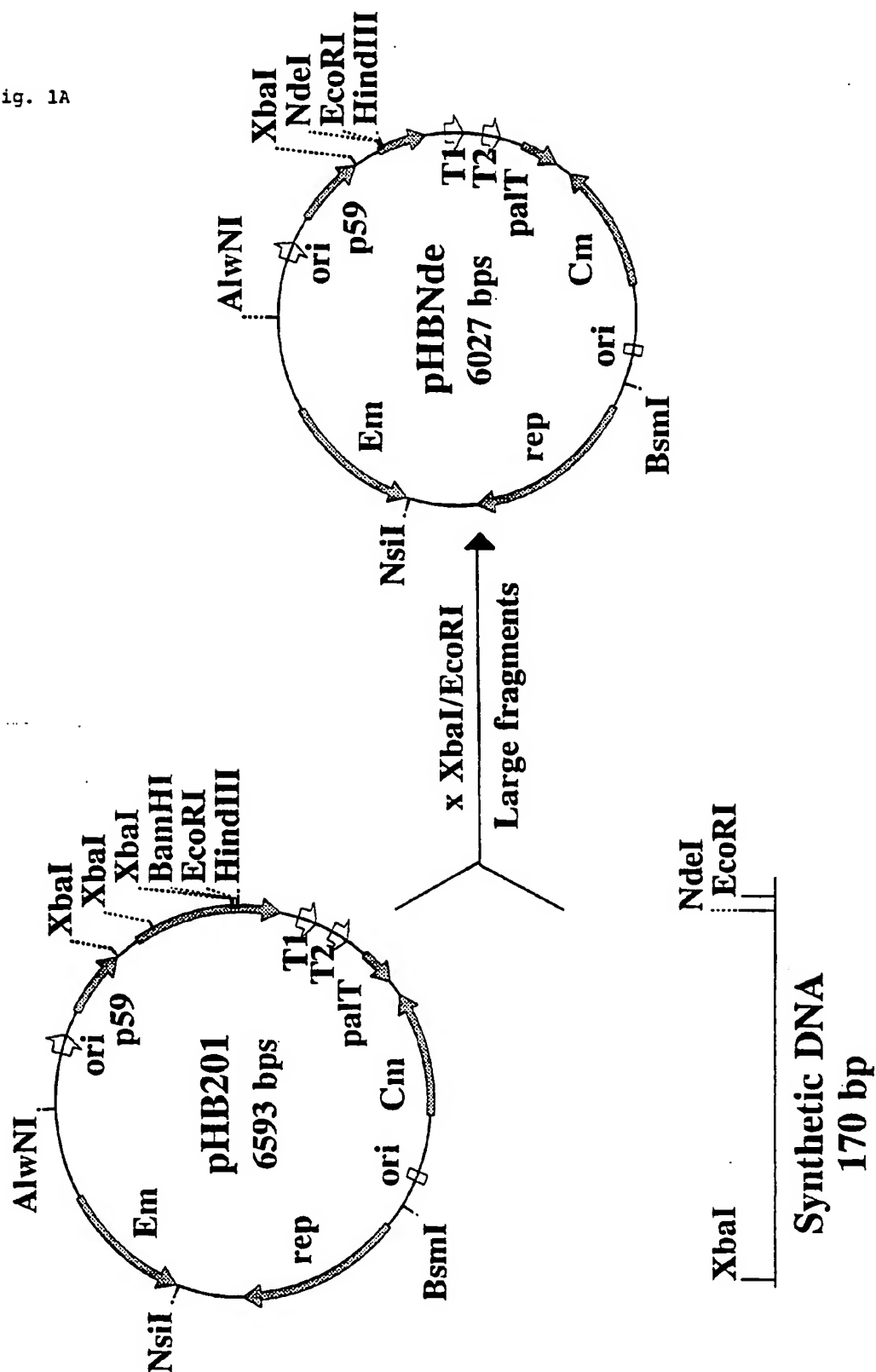
Ser Asp Leu Val Glu Lys Ala Asp Asp
  325

CLAIMS

1.   A proteinaceous substance comprising at least a
functional part of a secretion factor expressed by Gram-
positive bacteria encoded by the ftsY gene of said bacteria,
a representative of said gene being defined by the sequence
5   of seq. ID no. 7.

2.   A proteinaceous substance according to claim 1, which
is
at least partly encoded by the ftsY gene of a Bacillus
species.

10   3.   A proteinaceous substance according to claim 2, which
is
at least partly encoded by the ftsY gene of Bacillus
subtilis.

4.   A proteinaceous substance according to claim 3, which
15   is
at least partly encoded by the ftsY gene having the sequence
of seq. ID no. 7.

5.   A recombinant DNA molecule comprising at least a part
of
20   an ftsY gene encoding a chaperone protein of Gram-positive
bacteria, said part encoding at least a functional part of
said chaperone protein, whereby a representative of said
gene has the sequence of seq. ID no. 7.

6.   A recombinant DNA molecule according to claim 5,
25   comprising at least a part of the ftsY gene of a Bacillus
species.

7.   A recombinant DNA molecule according to claim 6,
comprising at least a part of the ftsY gene of Bacillus
subtilis.

30   8.   A recombinant vector comprising a recombinant DNA
molecule according to anyone of claims 5-7 and suitable
regulatory elements for replication and/or expression.

9.   A recombinant vector according to claim 8 further

comprising a gene encoding a protein of interest to be
secreted.

10.   A cell derived from a Gram-positive host cell
comprising

5   a recombinant DNA molecule according to anyone of claims 5-7
or a vector according to claim 8 or 9.

11.   A cell according to claim 10, further comprising a
vector comprising a gene encoding a protein of interest to
be secreted.

10   12.   A cell according to claim 10 or 11, further comprising
an enhanced amount of either or both Ffh and 7S scRNA.

13.   A cell according to claim 12 whereby the enhanced
amount of Ffh and/or scRNA are the result of overexpression
of the respective genes therefor.

15   14.   A cell according to claim 14 whereby said
overexpression is the result of the presence of at least one
vector comprising a gene ecoding Ffh or 7S scRNA.

15.   A method for enhancing the secretion of a protein of
interest from a Gram-positive micro-organism, comprising the

20   steps of providing said micro-organism with the capability
to over express the protein of interest, providing the
micro-organism with the capability of overexpressing a
proteinaceous substance according to claim 1, and culturing
said micro-organism under suitable conditions.

25   16. A method according to claim 15 wherein the capability to
overexpress the protein of interest is provided by a vector
according to claim 8 or 9.

17.   A method according to claim 15 wherein the capability
to overexpress proteinaceous substance according to claim 1

30   is provided by a vector according to claim 8 or 9.

18.   A method according to anyone of claims 15-17, further
comprising the step of providing the micro-organism with the
capability of overexpressing either or both 7S scRNA or Ffh
derived from the same or a different gram-positove organism.

35   19.   A method according to claims 15-18, wherein the gram-
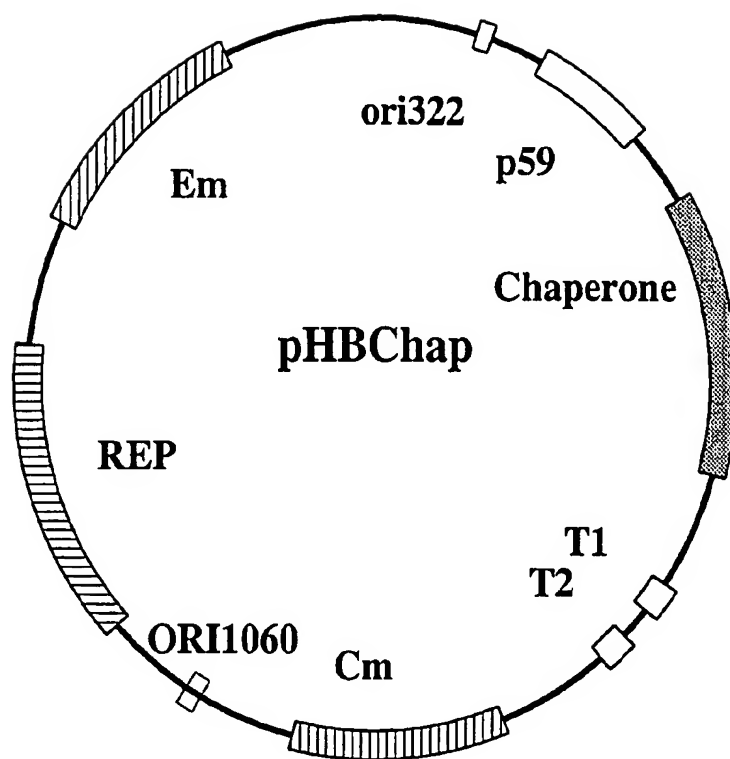positive organism is a Bacillus species.

20. A method according to claim 19, wherein the Bacillus
species is selected from the group comprising Bacillus
subtilis, Bacillus amyloliquefaciens, Bacillus
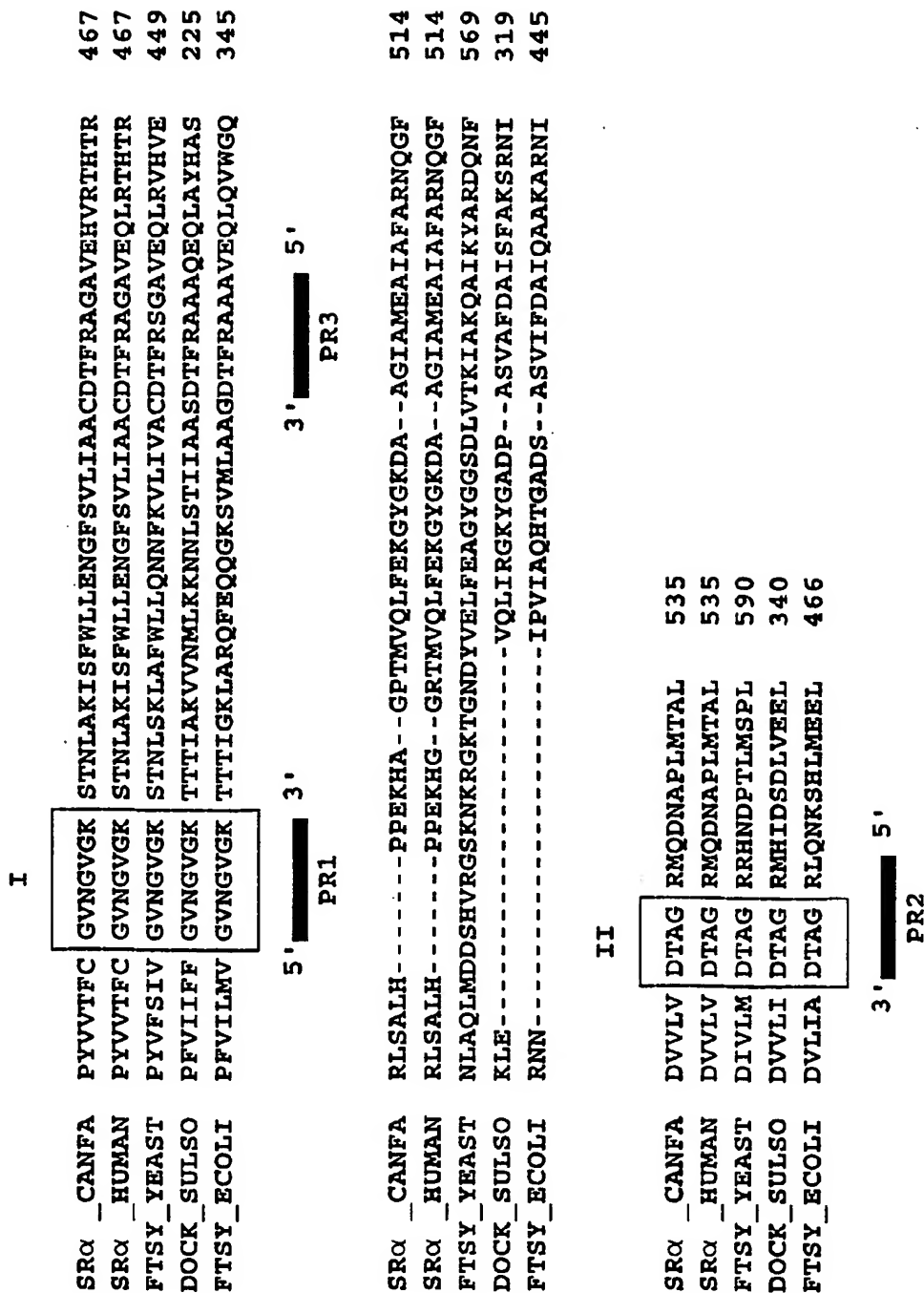alcalophilus, Bacillus centus and Bacillus
5   stearothermophilus.

Fig. 1A

Fig. 1B

Fig. 2A

I

```
SRα_CANFA    PYVVTFC GVNGVGK STNLAKISFWLLENGFSVLIAACDTFRAGAVEHVRTHTR   467
SRα_HUMAN    PYVVTFC GVNGVGK STNLAKISFWLLENGFSVLIAACDTFRAGAVEQLRTHTR   467
FTSY_YEAST   PYVFSIV GVNGVGK STNLSKLAFWLLQNNFKVLIVACDTFRSGAVEQLRVHVE   449
DOCK_SULSO   PFVIIFF GVNGVGK TTTIAKVVNMLKKNNLSTIIAASDTFRAAAQEQLAYHAS   225
FTSY_ECOLI   PFVILMV GVNGVGK TTTIGKLARQFEQQGKSVMLAAGDTFRAAAVEQLQVWGQ   345
```

5' 3'
PR1

3' 5'
PR3

```
SRα_CANFA    RLSALH------PPEKHA--GPTMVQLFEKGYGKDA--AGIAMEAIAFARNQGF     514
SRα_HUMAN    RLSALH------PPEKHG--GRTMVQLFEKGYGKDA--AGIAMEAIAFARNQGF     514
FTSY_YEAST   NLAQLMDDSHVRGSKNKRGKTGNDYVELFEAGYGGSDLVTKIAKQAIKYARDQNF    569
DOCK_SULSO   KLE-------------------------VQLIRGKYGADP--ASVAFDAISFAKSRNI  319
FTSY_ECOLI   RNN-------------------------IPVIAQHTGADS--ASVIFDAIQAAKARNI  445
```

II

```
SRα_CANFA    DVVLV DTAG RMQDNAPLMTAL   535
SRα_HUMAN    DVVLV DTAG RMQDNAPLMTAL   535
FTSY_YEAST   DIVLM DTAG RRHNDPTLMSPL   590
DOCK_SULSO   DVVLI DTAG RMHIDSDLVEEL   340
FTSY_ECOLI   DVLIA DTAG RLQNKSHLMEEL   466
```

3' 5'
PR2

Fig. 2B

```
SRα_CANFA   S T N L A K I S F W L L E N G F S V L I A A C   454
SRα_HUMAN   S T N L A K I S F W L L E N G F S V L I A A C   454
FTSY_YEAST  S T N L S K L A F W L L Q N N F K V L I V A C   433
DOCK_SULSO  T T T I A K V V N M L K K N G L S T I I A A S   209
FTSY_ECOLI  T T I G K L A R Q F E Q Q G K S V M L A A A G   329
              *   *         *               *
FTSY_BSUB   T T I G K L A H K M K Q E G K S V V L A A A G

Identity  :   3 ( 13.0%)
Similarity:  14 ( 60.9%)
```

```
 *  *  *   MOLECULE FEATURES   *  *  *


Type    Start   End   Compl  Name          Description


GENE       1    2286         'orf2         'orf2 = 3' part of ORF showing homology to DNA

                                           segragation genes like Yeast SMC1.

GENE     2306    3295         FtsY          FtsY

REGION   3294    3329         T             Terminator

GENE     3673    3332 (C)     orf3          orf3

REGION   3904    3939         P             promoter orf2-ffh operon

GENE     3992    4324         orf1          orf1

GENE     4338    4370         ffh'          ffh'= 5' part of ffh gene
```

FIG. 3

NAME: FTSY_BACSU_TO   4370 BPS DNA

                        * * * S E Q U E N C E * * *

      1 CTGCAGGAAC GGCATGATAT TTCTGCGCGT AAAGCCGCAT GTGAAACGGA ATTTGCCCGA

     61 ATTGAGCAGG AGATTCACAG TCAAGTCGGT GCATATCGTG ATATGCAGAC AAAATATGAG

    121 CAGAAAAAGC GCCAATACGA AAAAAATGAA TCCGCTCTGT ATCAGGCATA CCAATACGTT

    181 CAGCAAGCGA GATCAAAAAA GGACATGCTT GAGACGATGC AGGGAGATTT CTCCGGCTTT

    241 TATCAAGGTG TTAAAGAAGT GCTGAAAGCG AAAGAGCGCC TTGGCGGAAT TCGCGGAGCG

    301 GTTCTTGAGC TGATTTCTAC AGAACAGAAG TATGAAACGG CCATTGAAAT AGCGCTCGGC

    361 GCTTCTGCTC AACACGTCGT GACCGACGAT GAACAATCTG CCCGCAAAGC GATTCAATAT

    421 TTAAAGCAGA ATTCCTTCGG CCGGGCGACG TTTCTGCCTC TTTCTGTTAT TAGAGACCGC

    481 CAGCTTCAAA GCCGTGACGC GGAAACAGCC GCCCGGCATT CATCATTTCT CGGGGTTGCC

    541 AGTGAACTTG TCACATTTGA TCCTGCGTAT CGAAGCGTCA TCCAGAATCT TCTTGGAACC

    601 GTTCTGATCA CAGAGGACTT AAAGGGTGCA AACGAGCTTG CGAAGCTTCT CGGGCACCGG

    661 TACCGCATCG TAACCCTTGA GGGAGATGTT GTGAACCCCG GTGGTTCAAT GACGGGCGGC

    721 GCGGTTAAAA AGAAAAATAA CTCCCTCCTT GGAAGAAGCC GGGAGCTAGA AGATGTGACG

    781 AAACGGCTCG CTGAAATGGA AGAGAAAACG GCACTGCTTG AACAAGAGGT CAAAACCCTT

    841 AAGCACTCCA TTCAGGATAT GGAGAAAAAA CTGGCTGACT TAAGAGAAAC CGGGGAAGGC

    901 TTAAGGTTAA AGCAGCAGGA TGTGAAAGGC CAGCTGTACG AACTTCAAGT TGCCGAGAAA

    961 AATATCAATA CCCATTTAGA GCTCTATGAT CAAGAAAAAT CTGCTCTGTC AGAAAGCGAT

   1021 GAAGAGAGAA AAGTGCGCAA ACGCAAGCTT GAAGAAGAGC TCTCTGCCGT ATCTGAAAAG

   1081 ATGAAACAGC TTGAAGAGGA CATAGACAGA CTGACAAAAC AAAAACAAAC GCAATCCTCA

   1141 ACGAAAGAGT CTCTCTCCAA CGAGCTGACT GAGCTGAAGA TCGCAGCGGC CAAAAAAGAG

   1201 CAGGCTTGCG AGGGGGAAGA GGACAACCTT GCCAGATTAA AGAAAGAGCT CACTGAAACA

   1261 GAGCTTGCGT TAAAAGAAGC AAAAGAAGAC TTAAGCTTCT TAACGTCAGA GATGTCATCT

   1321 AGCACCAGCG GCGAAGAAAA GCTGGAAGAA GCAGCAAAAC ATAAATTGAA TGACAAAACG

   1381 AAAACGATCG AACTGATTGC ATTAAGGCGC GATCAGCGCA TCAAGCTTCA GCATGGGCTT

                        SUBSTITUTE SHEET (RULE 26)

```
1441  GATACGTATG AGCGTGAGCT GAAAGAAATG AAACGCCTGT ATAAACAAAA AACAACGCTC

1501  TTAAAAGACG AAGAAGTCAA ACTTGGACGA ATGGAAGTCG AGCTTGATAA TTTACTCCAA

1561  TACTTACGGG AGGAATACAG CTTGTCCTTT GAGGGGGCAA AAGAGAAATA TCAGCTTGAA

1621  ACTGATCCAG AGGAAGCCAG AAAGCGCGTG AAGCTGATTA AACTCGCAAT TGAAGAGCTG

1681  GGTACCGTGA ACCTCGGAAG CATAGATGAG TTTGAGAGGG TCAACGAACG GTACAAGTTT

1741  CTGTCGGAAC AAAAAGAAGA TTTAACAGAA GCGAAAAATA CCTTGTTCCA AGTGATTGAA

1801  GAAATGGATG AAGAAATGAC GAAGCGCTTT AACGACACAT TCGTCCAAAT CCGCTCACAC

1861  TTTGATCAAG TTTTCCGCTC CTTATTCGGA GGAGGGCGAG CTGAACTGAG GCTCACCGAT

1921  CCTAACGACT CCTCATCAGG ATCGAGATTA TCGCTCAGCC GCCGGGGAAA AACTCCAAAC

1981  TTTAACCTCC TGTCAGGCGG AGAGCGTGCG CTTACTGCTA TAGCGCTCTT ATTCTCAATC

2041  CTAAAGGTTC GTCCAGTGCC GTTTTGCGCC CTTGACGAAG TAGAGGCTGC GCTCGACGAA

2101  GCGAATGTGT TCCGATTTGC GCAGTACTTA AAAAAATACA GCAGCGATAC TCAGTTTATT

2161  GTGATTACCC ACAGAAAAGG AACGATGGAG GAAGCGGATG TGCTTTATGG CGTAACCATG

2221  CAGGAATCCG GTGTTTCCAA GGTAATTTCA GTTAAGCTGG AAGAAACAAA AGAATTCGTT

2281  CAGTAACGAG GAAAGAGGTT AAAAGATGAG CTTTTTTAAA AAATTAAAAG AGAAAATCAC

2341  AAAACAGACA GATTCCGTAT CTGAAAAGTT TAAGGATGGC CTTGAAAAAA CAAGAAACTC

2401  CTTTCAAAAC AAAGTGAATG ATCTTGTATC CCGTTACCGT AAAGTGGATG AGGATTTCTT

2461  CGAAGAGCTT GAAGAGGTTC TTATCAGCGC GGATGTCGGT TTTACAACCG TTATGGAATT

2521  AATAGATGAG CTGAAAAAAG AAGTCAAACG CAGAAATATT CAAGATCCAA AGGAAGTCAA

2581  GTCAGTGATT TCTGAGAAAC TGGTCGAGAT TTATAACAGC GGAGATGAGC AAATTTCAGA

2641  ACTGAACATC CAGGATGGGC GTTTAAACGT AATCCTTCTG GTAGGTGTAA ACGGCGTCGG

2701  GAAAACAACA ACGATCGGAA AGCTTGCTCA TAAAATGAAA CAAGAAGGAA AATCTGTTGT

2761  ACTTGCCGCC GGAGACACTT TTAGAGCGGG AGCCATTGAA CAGCTGGAAG TATGGGGAGA

2821  GCGTACAGGA GTGCCTGTCA TTAAGCAGAC GGCAGGAAGC GATCCGGCGG CTGTCATCTA

2881  CGATGCTGTT CATGCTGCGA AAGCAAGAAA TGCCGATGTA TTAATTTGTG ATACGGCAGG

2941  GCGTCTCCAA AACAAAGTAA ATCTCATGAA AGAGCTTGAA AAAGTAAAAC GTGTTATCGA

3001  AAGAGAAGTT CCTGAAGCTC CGCATGAGGT GCTGCTTGCC CTTGATGCCA CGACCGGCCA

3061  AAATGCAATG GCTCAGGCAA AAGAATTCTC TAAAGCAACA AATGTTACCG GCATTGCTTT

3121  AACGAAGCTT GACGGTACGG CAAAAGGCGG TATCGTCCTT GCGATTCGCA ACGAGCTTCA

3181  CATCCCGGTT AAACTAGTCG GTTAGGAGA AAAAGTTGAT GACCTTCAGG AATTTGATCC

3241  AGAATCCTAT GTGTACGGAC TCTTTTCAGA TTTAGTGGAA AAAGCCGACG ATTAAGAAAA

3301  AGGCCCCAAC ATCTTGGGGC CTTTTTCTTT TTTATCTTCT TACTTGATAG GCGAAATGAT
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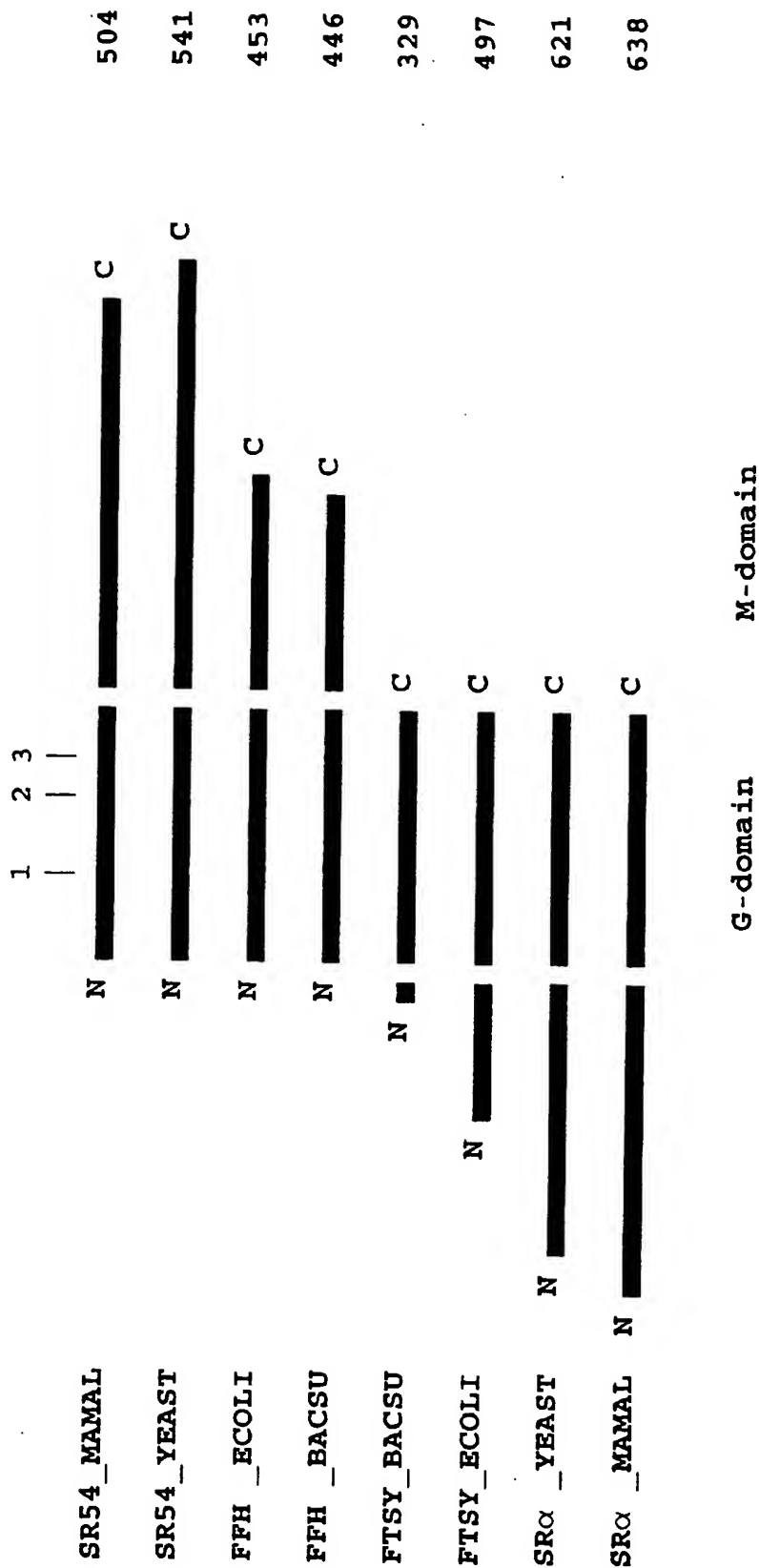
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3361  AAAGGCTGTT ATCAGTGGAT ACCAGTCTTG ACTCACCAGA AAAAACTCTG AATGGGATGA'

3421  TGTCATAGTA ATGAACGGAA ACAGATGTGT AATACGTATA GTAACCAGCA GCTGGCCCCA

3481  AATACATTGG AACCTCAAAT GTTCCGTTTG CATCAGTCGT TCCTGAAGCA GTTTGTGTTG

3541  TGTTTCCGAC CTTCGTGTCC GCTTCAAATC TTACGGGCGC GTTTGGCACT GGCTGTCCGT

3601  TTTGGTCGAG TAATTTGCCG CTTACTGTAA TATTGTACTT GACTCGCAAT ATTGACCTTG

3661  TCCGTAATTG ATTTTACCGT ATACCCCTCC ATCTGTGCTG ATATTTGTGA TTGAGGCCTT

3721  ATAAGGTGCC TCAGCAGCGT CTGCTTGCTG TGCCGGGAAA CCTATTGTAA ACAGGGCTGC

3781  CAGACATAAC ATAAACAATA AACCGATTTT TTTCATAAAA ATCCTCCTTA AAATAGGGTT

3841  CATATACAAT ATCGGAATAA ATTGGATGAT ATTTAGCGTA TTTTGGAAAA GTTAATCGCC

3901  GCTTTGACAA GATAAAAACT TGACAGTGTC ATTAAAACCG TGTAAACTAA GTTATCGTAA

3961  AGGGATTTGA CTTAACAAGG GGAGAGCTCA AATGTCACTC GAAAAGACAA GCAGAATGAA

4021  TTATCTGTTT GATTTTTATC AGCCGTTGTT GACGTCAAAA CAGAAGAGCT ATATGTCGCT

4081  TTATTATTTG GACGATTTCT CCCTAGGCGA AATAGCCGAA GAATATGAGG TTTCAAGACA

4141  AGCTGTTTAT GATAACATCA AACGAACAGA AGCAATGCTT GAACAATATG AAGAAAAGCT

4201  GCTCCTTTTG AAAAAGTTTC AGGAGCGTAA AGAGATGTTT AATAAGCTGA AGGAGCTTGC

4261  TTCCGGTTCA AAAGAAGAGG AAGAAATTAC AGCTCTGATT GAAGCGCTTG AGAAATTAGA

4321  TTAGGAGGCG GCAAACTATG GCATCTGAAG GATTAGCCGA CCGACTGCAG
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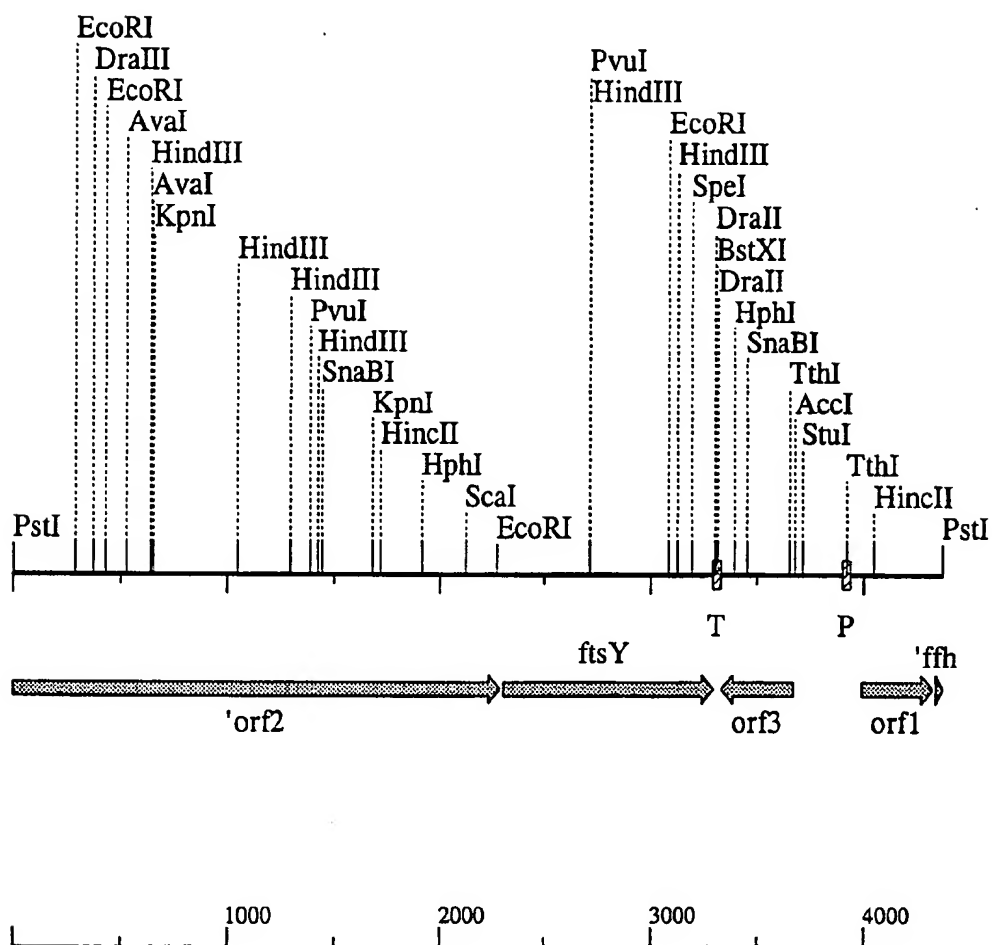
Fig. 4

Schematic alignment of *SRP54*, *Ffh*, *FtsY* and *SR*α
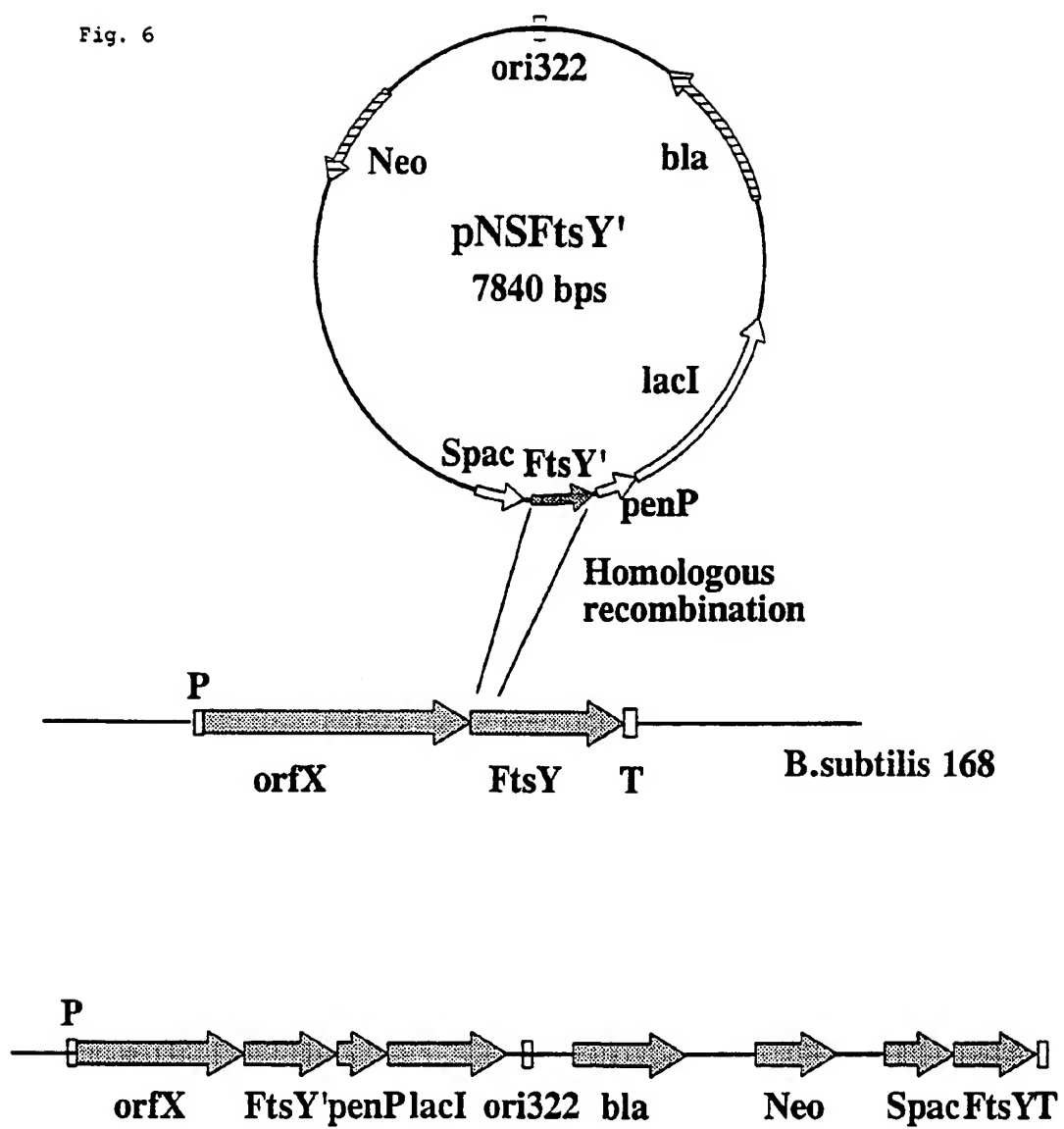

	504	541	453	446	329	497	621	638

SR54_MAMAL
SR54_YEAST
FFH_ECOLI
FFH_BACSU
FTSY_BACSU
FTSY_ECOLI
SRα_YEAST
SRα_MAMAL

G-domain   M-domain

Fig. 5   **B. subtilis chromosomal ftsY region**

(4370 bps)

Fig. 6

Fig. 7

Digest
with HpaI
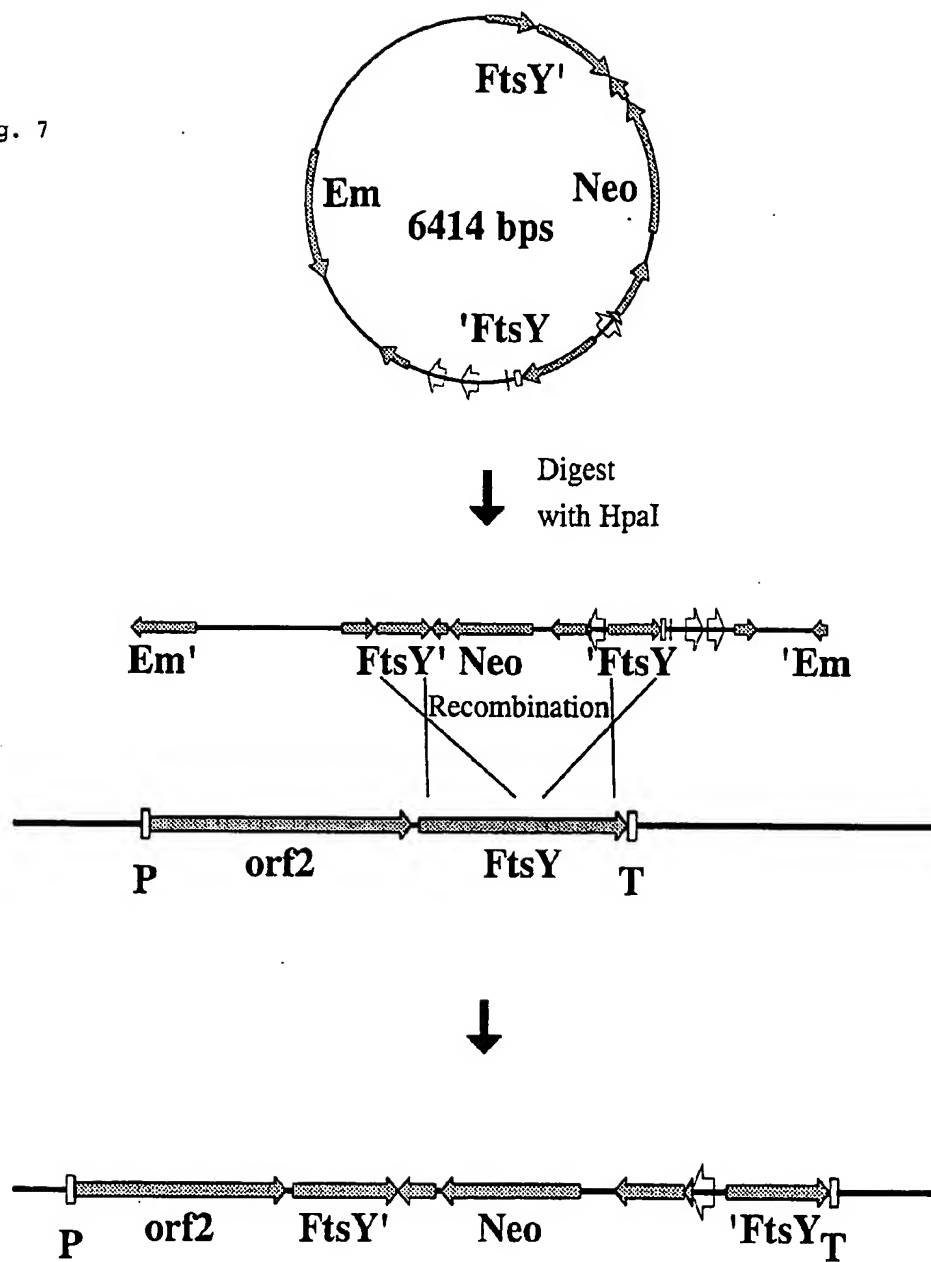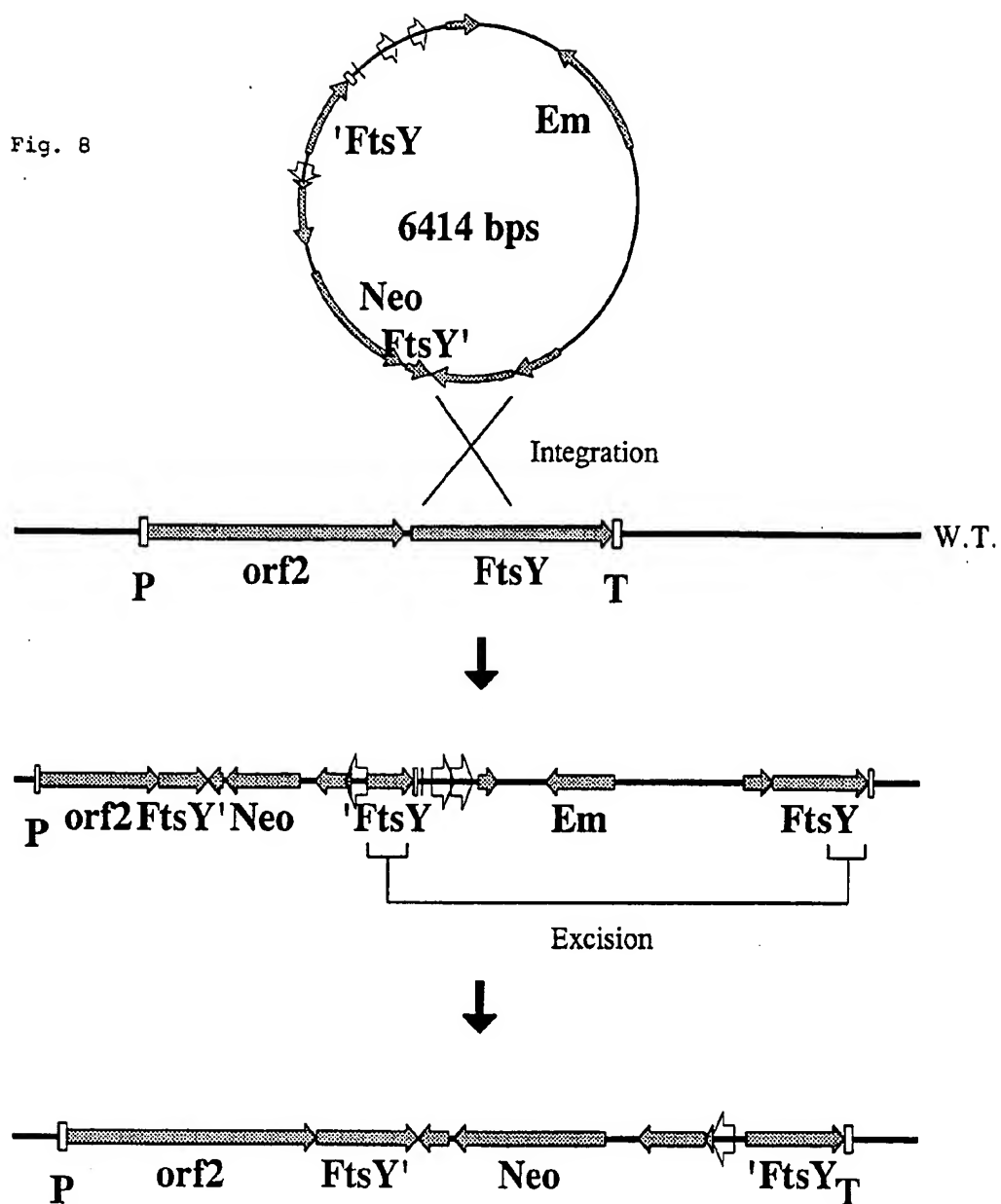
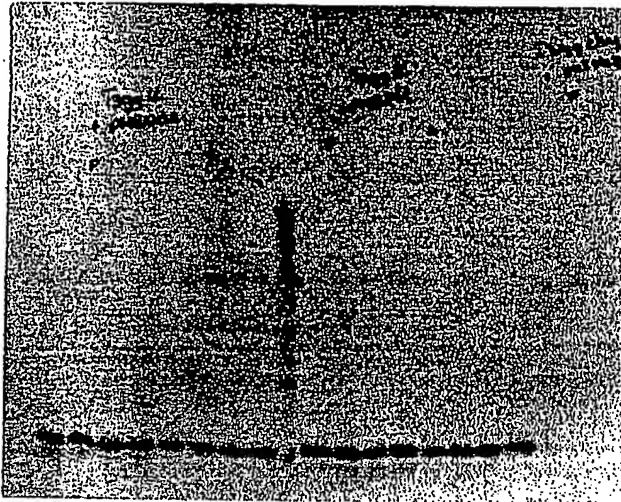Construction of ftsY disruption strain.

Fig. 8

Construction of ftsY disruption strain.

14/14

Fig. 9

0"15"30"60"   0"15"30"60"0"15"30"60"
      0"15"30"60"
pellet   sup   pellet   sup

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6   C12N15/31   C07K14/32   C12N1/21   C12N15/67   C12N15/75
//(C12N1/21,C12R1:07)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   C12N   C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. BACTERIOL., vol. 175, 1993, pages 4885-4894, XP000608463 HONDA K. ET AL.: "Cloning and characterization of a Bacillus subtilis gene encoding a homolog of the 54-kilodalton subunit of mammalian signal recognition particle and Escherichia coli Ffh" cited in the application see page 4892, right-hand column, last paragraph; figures 3,9 --- -/--	1-20

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 October 1996	05. 11. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Espen, J

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DNA RES, 1995, 2 (2) P95-100, JAPAN, XP000607148 OGURO A ET AL: "srb: a Bacillus subtilis gene encoding a homologue of the alpha-subunit of the mammalian signal recognition particle receptor." see figures 2,4 ---	1-20
Y	EMBO J, MAY 15 1994, 13 (10) P2289-96, ENGLAND, XP000608279 LUIRINK J ET AL: "An alternative protein targeting pathway in Escherichia coli: studies on the role of FtsY." cited in the application see the whole document ---	1-20
Y	NATURE, FEB 17 1994, 367 (6464) P657-9, ENGLAND, XP000608227 MILLER JD ET AL: "Interaction of E. coli Ffh/4.5S ribonucleoprotein and FtsY mimics that of mammalian signal recognition particle and its receptor [see comments]" see the whole document ---	1-20
Y	CELL, 77 (6). 1994. 787-790., XP000608274 WOLIN S L: "From the elephant to E. coli: SRP-dependent protein targeting" see the whole document ---	1-20
A	QUAX W J ET AL: "Correct secretion of heterologous proteins from Bacillus licheniformis" , 0 (0). 1993. 143-147. XP000607472 in 'Industrial Microorganisms: Basic and Applied Molecular Genetics', BALTZ R H ET AL (EDS) -----	

2